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(54) Title: INTERLEUKIN 1 β PROTEASE (57) Abstract There is disclosed an isolated polypeptide and derivatives thereof having protease biological activity for human precursor IL-1 β and for a substrate comprising: R ₁ -Asp-R ₂ -R ₃ , Wherein R ₁ and R ₃ are independently and D or L isomer amino acid, R ₂ is Ala or Gly, and wherein the specific protease cleavage site is between Asp and R ₂ . Inhibitor compounds, compositions and methods for inhibiting Interleukin 1 β protease activity are disclosed. The inhibitor compounds comprise an amino acid sequence of from 1 to about 5 amino acids having an N-terminal blocking group and C-terminal Asp residue connected to an electronegative leaving group, wherein the amino acid sequence corresponds to the sequence Ala-Tyr-Val-His-Asp.		

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TITLE**INTERLEUKIN 1B PROTEASE**

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TECHNICAL FIELD OF THE INVENTION

This invention relates to an interleukin 1B protease enzyme (IL-1B pro) having biological activity to cleave inactive precursor interleukin-1B (IL-1B) polypeptides into active mature IL-1B polypeptides. More specifically, the invention provides an isolated IL-1B pro polypeptide and derivatives thereof that are capable of cleaving a particular amino acid sequence, including the amino acid sequence at the N-terminus of human IL-1B. The present invention further provides a group of compounds that can inhibit IL-1B pro activity and thereby function as IL-1 antagonists.

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BACKGROUND OF THE INVENTION

Interleukin 1B (IL-1B) is a 17.5 kDa polypeptide hormone synthesized and secreted by stimulated monocytes. The initial translation product of IL-1B is a larger 31 kDa biologically inactive precursor polypeptide. The N-terminus of biologically active, mature IL-1B derived from human activated monocytes has been characterized by an N-terminal amino acid sequence beginning with Ala-Pro. See, for example, European Patent Application EP-A-0165654 and March et al. *Nature (London)* 315: 641-47 (1985) for sequence information of human IL-1B.

The N-terminal Ala residue of human mature IL-1B is in the 117 position and an Asp residue is in the 116 position counting from the N-terminus of human precursor IL-1B polypeptide. Mature IL-1B consists of the C-terminal 153 residues of the precursor polypeptide.

Maturation and release of mature IL-1B from macrophages does not proceed by conventional means normally associated with most secretory proteins, because the precursor IL-1B polypeptide lacks a hydrophobic signal sequence. Further, IL-1B is not associated with a membrane-bound compartment in monocytes. (Singer et al. *J. Exp. Med.* 167:389-407 (1988)). Most secretory proteins are characterized by the presence of a hydrophobic stretch of amino acids called a signal sequence. The signal sequence directs the translocation of the protein across the membrane of the endoplasmic reticulum during protein synthesis. The protein is subsequently ushered out of the cell via exocytosis. Most secreted proteins have a signal sequence at the amino terminal that is removed upon translocation. Other proteins, such as ovalbumin, have an internal signal sequence that is not removed upon translocation. Both precursor forms of IL-1 α and IL-1B (March et al.) lack any region (either amino terminal or internal) with sufficient hydrophobicity and length to qualify as a signal sequence.

A further indication of an unusual maturation pathway for IL-1 β is the absence of a pair of basic amino acids near the N-terminus of the mature polypeptide. The amino acid sequence Tyr-Val-His-Asp- precedes the N-terminal Ala-Pro of the mature human IL-1 β polypeptide. Moreover, Young et al. *J. Cell Bio.* 107:447-56 (1988) found that fibroblasts
5 transfected with cDNA coding for precursor IL-1 β were unable to process the precursor polypeptide into mature IL-1 β . Instead, the transfected fibroblasts produced high levels of inactive precursor polypeptide. The results reported by Young et al. are consistent with other reports for other cell types, including T cells, epidermal cells and B cells.

Hazuda et al. *J. Biol. Chem.* 263:8473-79 (1988) have reported that both the
10 precursor and mature forms of IL-1 β appear in the supernatants of activated monocytes with little or no preference. Hazuda et al. suggest that IL-1 β processing is "intimately coordinated" with secretion.

There have been several attempts to characterize or isolate the system responsible for processing IL-1 β from its translated precursor form to its active mature form. Black et al. *J. Biol. Chem.* 263:9437-42 (1988) [Black et al. I] suggests that the cleavage pattern of
15 precursor IL-1 β is effected by myeloid cell membranes and results from the action of a plurality of proteases which act as an IL-1 β processing system. A subsequent article by Black et al. *J. Biol. Chem.* 264:5323-26 (1989) [Black et al. II] describes a single protease that cleaves IL-1 β between His¹¹⁵ and Asp¹¹⁶, one residue upstream from the the N-
20 terminal Ala¹¹⁷ of mature IL-1 β . Thus, the protease described in Black et al. II generates a form of IL-1 β one amino acid longer than the mature IL-1 β purified from monocyte cultures. Black et al. II suggests that there may be an aminopeptidase in human blood that removes the N-terminal aspartate residue to complete the processing.

Kostura et al. *Proc. Nat Acad Sci. USA* 86:5227-31 (July, 1989) refers to a
25 protease with a similar cleavage pattern but "qualitatively different" from the protease described in Black et al. II. The Kostura et al. protease is characterized as being located in cytosol of monocytic cells. However, Kostura et al. did not further define or isolate the responsible polypeptide.

Finally, Black et al. *FEBS Lett.* 247:386-90 (April, 1989) [Black et al. III] refers to
30 a protease that generates mature IL-1 β from the precursor polypeptide and is characterized by being inhibited by iodoacetate and N-ethylmaleimide. Black et al. III attempted to purify their protease approximately 500 fold by a process starting by freeze-thawing cell lysates from THP-1 cells (ATCC) four times. Black et al. III centrifuged the lysates for 20 min at 36,590 X g. The supernatant was applied to a DEAE- Sephacel column equilibrated with
35 10 mM Tris-HCl (pH 8.1) and 5 mM dithiothreitol. The protease was eluted with 80-140 mM NaCl. The eluted material was diluted 1:5 with a buffer of 10 mM Tris-HCl (pH 8.1) and 5 mM dithiothreitol and applied to a procion red agarose column. The protease was

eluted with 0.5-0.8 M NaCl, concentrated 20-fold in a Centriprep-10 concentrator, and then subjected to gel filtration with Sephadex G-75. This procedure described in Black et al. III failed to demonstrate whether the protease activity was due to a single polypeptide or a group of processing enzymes.

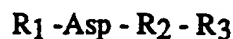
5 Therefore, there is a need in the art to obtain the isolated system or single protease polypeptide responsible for processing precursor IL-1 β into its mature and biologically active form. The protease functions as an IL-1 agonist to increase IL-1 biological activity *in vivo*. Moreover, the isolated protease will be useful for improving wound healing, treating arthritis, and treating or preventing the onset of autoimmune diseases, such as
10 insulin dependent diabetes melitus, lupus disorders, Graves' disease, Hashimotos disease, and the detrimental side effects of radiation treatment.

Further, isolation and characterization of the protease responsible for processing precursor IL-1 β into its biologically active form will aid in designing inhibitors for IL-1 β processing, because the availability of large quantities of IL-1 β pro will serve as a useful
15 screening vehicle for finding compounds having IL-1 antagonist activity. Such IL-1 antagonists or IL-1 β pro inhibitors should be useful for treating inflammation and transplantation rejection.

Other protease inhibitors have been described (for example, U.S. Patents 4,644,055, 4,636,492 and 4,652,552). However, protease inhibitors tend to be specific
20 for the protease they inhibit by having a chemical structure that mimics the substrate of a protease. Thus, none of the previously-described protease inhibitors would be expected to inhibit IL-1 β pro activity.

SUMMARY OF THE INVENTION

25 The present invention is directed to an isolated polypeptide having proteolytic activity for a specific protease cleavage site, wherein the protease activity is specific for a substrate peptide having an amino acid sequence comprising:



wherein R₁ and R₃ are independently any D or L isomer amino acid, R₂ is Ala or Gly, and
30 wherein the specific protease cleavage site is between Asp and R₂. Preferably, the substrate peptide is at least eight amino acids in length. The isolated polypeptide is called the interleukin 1 β protease (IL-1 β pro) because it cleaves precursor IL-1 β polypeptide to yield mature IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues. This region of precursor IL-1 β corresponds to a species within the genus of
35 protease cleavage sites described herein.

IL-1 β pro is further characterized by a cDNA and amino acid sequence in Figure 1 (Sequence I.D. No. 2). Full length (precursor) IL-1 β pro comprises 404 amino acids.

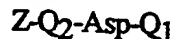
Purified IL-18 pro begins with the Asn Pro Ala Met Pro sequence beginning with amino acid 120. Based upon a molecular weight analysis, the approximate C-terminus of mature IL-18 pro is about amino acid 297. However, molecular weight determination indicates that the C-terminus of the mature IL-18 pro enzyme is from about amino acid 278 to about amino acid 315. The present invention comprises an isolated IL-18 pro polypeptide or a derivative, analog, or allelic variant thereof displaying IL-18 pro biological activity comprising proteolytically cleaving human precursor IL-18 polypeptide at a cleavage site between the Asp 116 and Ala 117 residues.

Figure 1 also shows a nucleotide sequence encoding a 404 amino acid polypeptide having IL-18 pro biological activity. The present invention further comprises an isolated DNA sequence encoding IL-18 pro or a derivative, analog or allelic variant thereof displaying biological activity to proteolytically cleave human precursor IL-18 polypeptide at a cleavage site between the Asp 116 and Ala 117 residues. The isolated DNA sequence is selected from the group consisting of the nucleotide sequences in Figure 1 beginning at nucleotide 1 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to a nucleotide from about 851 to about 962, DNA sequences which detectably hybridize to the Figure 1 sequence from nucleotide 1 to nucleotide 1232 and encode a polypeptide displaying biological activity to proteolytically cleave human precursor IL-18 polypeptide at a cleavage site between the Asp 116 and Ala 117 residues, and DNA sequences which, due to degeneracy of the genetic code, encode a mammalian IL-18 pro polypeptide encoded by any of the foregoing DNA inserts and sequences.

The present invention further comprises a recombinant expression vector comprising an isolated DNA sequence as described herein and a host cell which comprises the recombinant expression vector.

The present invention provides substituted peptide inhibitor compounds comprising an amino acid sequence of from 1 to about 5 amino acids, having an N-terminal protecting group and a C-terminal Asp residue connected to an electronegative leaving group. Preferably, the amino acid sequence corresponds to at least a portion of the amino acid sequence Ala-Tyr-Val-His-Asp.

The inhibitor compounds of the present invention have the formula:



where Z is an N-terminal protecting group; Q_2 is 0 to 4 amino acids such that the sequence $Q_2\text{-Asp}$ corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 2; and Q_1 comprises an electronegative leaving group. Z is preferably $C_1\text{-}C_6$ alkylketone, benzyl, acetyl, alkoxycarbonyl,

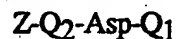
benzyloxycarbonyl or C₁-C₆ alkylcarbonyl. More preferably, Z is t-butoxycarbonyl (t-Boc), acetyl carbonyl or benzyloxycarbonyl (Cbz).

Q₁ is preferably C₁-C₃ alkyl, an aldehyde, diazomethyl ketone or halomethyl ketone. More preferably, Q₁ is an aldehyde or fluoromethyl ketone.

5 The present invention further provides reversible and irreversible IL-1 β pro inhibitors. Irreversible inhibitors are inhibitor compounds comprising an amino acid sequence of from 1 to about 5 amino acids having an N-terminal protecting group and a C-terminal Asp residue connected to a diazomethyl ketone or a halomethyl ketone, wherein the amino acid sequence corresponds to at least a portion of the sequence Ala-Tyr-Val-His-
10 Asp, residues 112 to 116 of sequence listing I.D. No. 1.

Reversible IL-1 β inhibitors are compounds comprising an amino acid sequence of from 1 to about 5 amino acids having an N-terminal protecting group and a C-terminal Asp residue connected to an aldehyde moiety, wherein the amino acid sequence corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence
15 listing I.D. No. 1.

The present invention also provides a method of inhibiting the physiological actions of interleukin 1 β in a mammal in need of such treatment, comprising administering to said mammal an effective amount of a compound of the formula:



20 where Z is an N-terminal protecting group; Q₂ is 0 to 4 amino acids such that the sequence Q₂-Asp corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 2; and Q₁ comprises an electronegative leaving group.

In a preferred embodiment, Q₁ is a fluoromethyl ketone and inhibition is
25 irreversible. In another preferred embodiment, Q₁ is an aldehyde moiety and inhibition is reversible.

The present invention still further provides a pharmaceutical composition comprising a physiologically acceptable carrier and a compound comprising an amino acid sequence of from 1 to about 5 amino acids having an N-terminal protecting group and a C-terminal Asp residue connected to an electronegative leaving group, wherein said amino
30 acid sequence corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 1.

The present invention still further provides a method of treating inflammation associated with autoimmune disease in a mammal in need of such treatment comprising
35 administering to said mammal an effective anti-inflammatory amount of a compound comprising an amino acid sequence of from 1 to about 5 amino acids having an N-terminal protecting group and a C-terminal Asp residue connected to an electronegative leaving

group, wherein said amino acid sequence corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 1.

The present invention further comprises antisense oligonucleotides that correspond to a sequence or antisense sequence of at least 15 nucleotides selected from the nucleotide sequence of Sequence ID No. 2 (Figure 1).

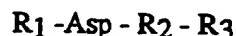
The present invention further comprises a method for treating arthritis, a method for treating an autoimmune disease in a susceptible individual, a method for improving wound healing, and a method for reducing the detrimental side effects of radiation treatment. All of the methods comprise administering a therapeutically effective amount of an isolated IL-18 protease or a biologically active derivative thereof in a suitable pharmaceutical carrier.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the DNA and corresponding amino acid sequence for a polypeptide having IL-18 pro biological activity and corresponding to human mature IL-18 pro or a biologically active fragment thereof.

DETAILED DESCRIPTION OF THE INVENTION

Utilizing polymerase chain reaction (PCR) techniques, we have isolated, purified, characterized, and expressed a mammalian IL-18 pro polypeptide and active fragments thereof. The availability of abundant quantities of a recombinant IL-18 pro enzyme has further allowed us to find inhibitor compounds capable of inhibiting IL-18 pro activity and thereby function as IL-1 antagonists. Further, use of IL-18 pro has IL-1 agonist activity. Thus, the invention relates to mammalian IL-18 pro polypeptides, derivatives, analogs and allelic variants thereof having proteolytic activity for a substrate peptide having an amino acid sequence comprising:



wherein R₁ and R₃ are independently any D or L isomer amino acid, R₂ is Ala or Gly, and wherein the specific protease cleavage site is between Asp and R₂. Preferably, the substrate peptide is at least eight amino acids in length. Most preferably, R₂ is Gly.

Mammalian IL-18 pro is preferably a human IL-18 pro and has substrate specificity for a substrate peptide having the amino acid sequence described herein. Preferably, the human IL-18 pro polypeptide or derivative thereof is a polypeptide having biological activity that cleaves human precursor IL-18 polypeptide to yield human mature IL-18 polypeptide.

IL-18 pro is further characterized by the cDNA and amino acid sequence in Figure 1 (Sequence I.D. No. 2). Full length (precursor) IL-18 pro comprises 404 amino acids. Purified IL-18 pro begins with the Asn Pro Ala Met Pro sequence beginning with amino acid 120. Based upon a molecular weight analysis, the approximate C-terminus of mature

IL-1 β pro is about amino acid 297. However, the molecular weight determination indicates that the C-terminus of the mature enzyme is from about amino acid 278 to about amino acid 315. The present invention comprises an isolated IL-1 β pro polypeptide or a derivative, analog, or allelic variant thereof displaying biological activity comprising proteolytically cleaving human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues. The present invention comprises an isolated IL-1 β pro polypeptide or a derivative, analog, or allelic variant thereof displaying biological activity comprising proteolytically cleaving human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues. For the purposes of this application, the term "IL-1 β pro" shall encompass the amino acid sequence shown in Figure 1, plus all allelic variants, derivatives, analogs and fragments of this sequence that display IL-1 β pro biological activity.

IL-1 β pro biological activity is determined, for example, by assaying for IL-1 activity with a precursor IL-1 β polypeptide. Precursor IL-1 β is inactive, while mature IL-1 β is an active IL-1 polypeptide. A method for measuring IL-1 β pro activity is described in Black et al. II. Briefly, this method provides approximately five microliters of precursor IL-1 β (pIL-1 β) (10-50 μ g/ml prepared as described in Black et al. I) incubated with 10 μ l of IL-1 β pro polypeptide or another substance suspected of having IL-1 β pro biological activity. The incubation proceeds for approximately one hour at approximately 37°C and is terminated by addition of 15 μ l of 2 X SDS sample buffer followed by boiling for five minutes. The boiled sample is electrophoresed on a SDS-polyacrylamide gel and placed onto a Western blot using an IL-1 β C-terminal-specific monoclonal antibody, such as 16F5 described in Black et al. I.

Figure 1 also shows a nucleotide sequence encoding a 404 amino acid sequence having IL-1 β pro biological activity. The present invention further comprises an isolated DNA sequence encoding IL-1 β pro or a derivative, analog or allelic variant thereof displaying biological activity to proteolytically cleave human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues. The isolated DNA sequence is selected from the group consisting of the nucleotide sequences in Figure 1 beginning at nucleotide 1 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to a nucleotide from about 851 to about 962, DNA sequences which detectably hybridize to the Figure 1 sequence from nucleotide 1 to nucleotide 1232 and encode a polypeptide displaying biological activity to proteolytically cleave human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues, and DNA sequences which, due to degeneracy of the genetic code, encode a mammalian IL-1 β pro polypeptide encoded by any of the foregoing DNA inserts and sequences.

Inventive DNA sequences that detectably hybridize to the Figure 1 nucleotide sequence from nucleotide 1 to nucleotide 856, hybridize under conditions of high or severe stringency. Severe or high stringency conditions comprise, for example, overnight hybridization at about 68°C in a 6 X SSC solution followed by washing at about 68°C in a
5 0.6 X SSC solution.

The inhibitor compounds of the present invention are substituted compounds comprising an amino acid sequence of from 1 to about 5 amino acid residues having an N-terminal blocking group and a C-terminal Asp residue connected to an electronegative leaving group, wherein the amino acid sequence corresponds to at least a portion of the sequence Ala-
10 Tyr-Val-His-Asp, which sequence represents the sequence of residues 112 to 116 of precursor IL-1B.

The amino acid sequence of precursor IL-1B is presented in sequence listing LD. No. 2 taken from March et al., *Nature*, 315:641-647 (1985). Mature IL-1B is represented by the C-terminal 153 amino acid residues of precursor IL-1B. Thus, the N-terminal of IL-1B is the Ala
15 residue at position 117 of sequence listing LD. No. 1.

The naturally occurring amino acids are the L isomers and are so indicated without any isomeric (e.g., L or D) designation. The D isomers are so indicated.

As used herein, the phrase "corresponds to" means that a particular sequence may differ from the disclosed sequence by one or more conservative substitutions so long as such
20 substitutions do not materially alter the inhibitory activity of the compounds of the present invention.

Examples of substitutions that do not materially alter inhibitory activity are replacement of the Ala at position 112 of sequence listing LD. No. 1 with Ser or Gly; replacement of the Tyr at position 113 of sequence listing LD. No. 1 with Phe; replacement of the Val at position 114
25 of sequence listing LD. No. 1 with Leu, Ile or Met and replacement of the His at position 115 of sequence listing LD. No. 1 with Phe, Pro, a positively charged amino acid such as Lys, Arg, His or Tyr, or the use of D isomers.

The C-terminal amino acid residue of the compounds of the present invention is aspartic acid (Asp). Asp has a side chain of the formula $\text{CH}_2\text{-COOH}$. Preferably, the Asp side chain
30 carboxyl group is protected to facilitate synthesis of the compounds of the present invention.

Asp side chain protection groups include, for example, a benzyl, substituted benzyl, formyl methyl or t-butyl moiety. The benzyl substituents increase the acid lability of the Asp side chain protecting moiety. Exemplary substituted benzyls are 2,4,6-trimethyl benzyl and 4-methoxybenzyl.

35 In a preferred embodiment, the Asp side chain protecting moiety is connected to the Asp side chain via an ester linkage, which linkage is subject to cleavage by naturally occurring intracellular esterase enzymes. In this way, the protected Asp with high lipid solubility gains

access to a cell and is cleaved by an esterase to yield a charged, water soluble deprotected Asp that remains in the cytoplasm where IL-1 β pro is predominantly located.

As used herein, the phrase "N-terminal blocking group" refers to chemical groups attached to the amino group of the N-terminal amino acid residue of the sequences of the present invention. Such blocking groups are well known and readily apparent to those of skill in the art. The Peptides, ed. by Gross and Meienhofer, Academic Press, New York, pp. 3-81 (1981). N-terminal blocking groups have been utilized with other types of protease inhibitors. See, e.g., U.S. Patent Nos. 4,652,552 and 4,636,492.

As used herein, the phrase "electronegative leaving group" refers to chemical groups susceptible to nucleophilic attack by an amino acid residue in the enzyme active site, thus modifying IL-1 β pro such that IL-1 β pro cannot interact with and cleave precursor IL-1 β .

The inhibitor compounds of the present invention inhibit the catalytic activity of IL-1 β pro in a reversible or an irreversible fashion. As used herein, "irreversible" means the formation of a covalent bond between the enzyme and the inhibitor.

The reversibility of IL-1 β pro activity is a function of the electronegative leaving group. When the electronegative leaving group is a diazoalkyl ketone, the inhibition of IL-1 β pro is irreversible and the compound is an irreversible inhibitor. When the electronegative leaving group is an aldehyde, the inhibition of IL-1 β pro is reversible and the compound is a reversible inhibitor.

The inhibitor compounds of the present invention have the formula:



where Z is an N-terminal blocking group; Q_2 is 0 to about 4 amino acids such that the sequence Q_2 -Asp corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 1; and Q_1 is an electronegative leaving group.

In a preferred embodiment, Z is C_1 - C_6 alkyl, benzyl, acetyl, C_1 - C_6 alkoxy carbonyl, benzyloxy carbonyl or C_1 - C_6 alkyl carbonyl. As used herein, "alkyl" refers to linear or branched chains having 1 to 6 carbon atoms, which may be optionally substituted as herein defined. Representative alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, pentyl, hexyl and the like. In a more preferred embodiment, Z is t-butoxycarbonyl (t-Boc), acetyl or benzyloxy carbonyl (Cbz).

Q_2 is preferably 1 amino acid. Preferably Q_2 is His, Phe, Pro, or Tyr. Most preferably, Q_2 is His or Phe.

Q_1 is preferably an aldehyde, a diazoalkyl ketone or a haloalkyl ketone. As used herein in reference to electronegative leaving groups, "alkyl" refers to linear or branched chain radicals having 1 to 3 carbon atoms, which may be optionally substituted as herein defined. Representative alkyl groups include methyl, ethyl, propyl and the like. More preferably, Q_1 is an aldehyde or fluoromethyl (CH_2F) ketone.

The inhibitor compounds of the present invention are made by techniques generally corresponding to methods known and readily apparent to those of skill in the art. See, e.g., Kettner, C.A. et al., Arch. Biochem. Biophys., 162:56 (1974); U.S. Patent No. 4,582,821; U.S. Patent No. 4,644,055; Kettner, C.A. et al., Arch. Biochem. Biophys., 165:739 (1974);
5 Dakin, H.D. and West, R., J. Biol. Chem., 78:91 (1928); Rasnick, D., Anal. Biochem., 149:461 (1985).

Inhibitor compounds having a fluoromethyl electronegative leaving group are preferably synthesized by the Rasnick procedure.

10 Inhibitor compounds having a non-fluoro, haloalkyl ketone electronegative leaving group are synthesized in accordance with the Kettner procedure. An N-blocked amino acid or peptide is reacted with N-methylmorpholine and an alkyl, non-fluoro haloformate to generate a peptide-acid anhydride. The anhydride is then reacted with a diazoalkane in an inert, aprotic solvent to form a peptide-diazomethane ketone. The diazomethane ketone is then reacted with an anhydrous solution of HCl, HBr or HI to produce the desired N-blocked, C-terminal
15 haloalkyl ketone peptide or amino acid.

Inhibitor compounds having a fluoroalkyl ketone electronegative leaving group are synthesized in accordance with a Rasnick procedure. An N-blocked peptide is reacted with fluoroacetic anhydride and a trialkylamine in an organic solvent to form a peptide-anhydride. The anhydride is then reacted with a catalyst such as 4-dimethylaminopyridine and the reaction
20 mixture maintained at about 25° C for about two hours to allow for CO₂ evolution. The reaction mixture is then extracted with an organic solvent and the organic phase washed and dried. The organic solvent is removed to form an oil, which is then applied to a silica gel column. The N-blocked, fluoroalkyl ketone peptide is then eluted from the gel and purified.

Inhibitor compounds having a fluoroalkyl ketone electronegative leaving group can be extended in the N-terminus direction by removing the N-terminal blocking group and coupling
25 the deprotected compound with other protected amino acids. Bodanszky, *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin (1984). Alternatively, deprotected compounds are acetylated to yield compounds having an N-terminal acetyl protecting group. Stewart et al., *Solid Phase Peptide Synthesis*, Pierce Chemical Co., Rockford, IL. (1984).

30 The inhibitor compounds of the present invention are useful in inhibiting the physiological actions of interleukin 1 β by preventing formation of biologically active IL-1 β . The inhibition of IL-1 β pro results in a decrease in active IL-1 β levels and a concomitant increase in precursor IL-1 β , which compound is biologically inactive.

The inhibitor compounds of the present invention are also useful in treating
35 dysfunctional states, such as autoimmune disease-associated inflammation, often mediated by increased IL-1 activity.

Mammals needing treatment for an inflammatory disorder or prevention of an autoimmune condition are administered effective amounts of the compounds of this invention either alone or in the form of a pharmaceutical composition.

Pharmaceutical compositions of the present invention comprise physiologically acceptable carriers and compounds comprising amino acid sequences of from 1 to about 5 amino acids having an N-terminal blocking group and a C-terminal Asp residue connected to an electronegative leaving group, wherein said amino acid sequence corresponds to the sequence Ala-Tyr-Val-His-Asp, residues 112 to 116 of sequence listing I.D. No. 1.

Antisense oligonucleotides can be synthesized (by conventional phosphodiester techniques such as by Synthecell, Rockville, MD) that are complementary to unique species-specific coding regions of mRNA for IL-1 β pro. Examples of such regions include regions of at least 18 bases at the initiation codon (TACCGGCTGTTCCAGGAC or TACCTATTCTGGGCTCGA) complementary to bases 18-36 and 168 to 196, respectively in Figure 1, at the N-terminus of mature IL-1 β pro (TTGGGTTCGATACGGGTGT) complementary to bases 374 to 392 in Figure 1, at the approximate C terminus after protease cleavage (CACCACACCAAATTTCTA) complementary to bases 890 to 908 in Figure 1, or at a region immediately 5' to the termination codon (ATGGAGAAGGGTCCTGTA) complementary to bases 1205 to 1229 in Figure 1.

The primary amino acid structure of IL-1 β pro or its active fragment thereof may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants or derivatives. Covalent derivatives of IL-1 β pro are prepared by linking particular functional groups to IL-1 β pro amino acid side chains or at the N-terminus or C-terminus of the IL-1 β pro polypeptide. Other derivatives of IL-1 β pro within the scope of this invention include covalent or aggregative conjugates of IL-1 β pro or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated polypeptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the IL-1 β pro polypeptide which co-translationally or post-translationally directs transfer of the IL-1 β pro polypeptide from its site of synthesis to a site inside or outside of the cell membrane or wall (e.g., the yeast α -factor leader). IL-1 β pro polypeptide fusions can comprise polypeptides added to facilitate purification and identification of IL-1 β pro (e.g., poly-His). Further, the amino acid sequence of IL-1 β pro can be linked to the peptide Asp-Tyr-Lys-Asp-Asp-Asp-Lys (Hopp et al. *BioTechnology* 6:1204 1988), which is a highly antigenic sequence and provides an epitope reversibly bound by a specific monoclonal antibody to enable rapid assay and facile purification of the expressed recombinant polypeptide. This specific leader sequence is cleaved by bovine mucosal enterokinase at the residue immediately following

the Asp-Lys pairing. Moreover, fusion polypeptides having this leader sequence at its N-terminal may be resistant to degradation in *E. coli* host cells.

The present invention further includes IL-1 β pro polypeptides with or without associated native-pattern glycosylation. IL-1 β pro expressed in yeast or mammalian
5 expression systems (e.g., COS-7 cells) may be similar or significantly different in molecular weight and glycosylation pattern than the native human IL-1 β pro polypeptide. This depends upon the choice of expression system. Expression of IL-1 β pro polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Functional mutant analogs of human IL-1 β pro can be synthesized, for example,
10 with inactivated N-glycosylation sites by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. The IL-1 β pro derivatives can be expressed in homogeneous, reduced carbohydrate form using yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn- Φ - Ω where Φ is any amino acid except Pro and Ω is Ser or Thr. In this sequence,
15 carbohydrate residues are covalently attached at the Asn side chain.

IL-1 β pro analogs or derivatives may also be obtained by mutations of the IL-1 β pro DNA sequence. An IL-1 β pro mutant derivative, as referred to herein, is a polypeptide substantially homologous to IL-1 β pro but which has an amino acid sequence different from native IL-1 β pro because of a deletion, insertion or substitution.

20 IL-1 β pro is expressed from a mammalian gene, presumably encoded by one or more multi-exon genes. The present invention further includes alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share regions of identity or similarity with the cDNA's disclosed herein.

25 Bioequivalent analogs of IL-1 β pro polypeptides (defined as polypeptides having IL-1 β pro biological activity) may be constructed, for example, by making various substitutions of amino acid residues or sequences, or by deleting terminal or internal residues or sequences not needed for biological activity. For example, Cys residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular
30 disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions are made conservatively by substituting an amino acid having physiochemical characteristics resembling those of the replaced residue. Further substitutions may be made outside of the "core" sequence needed
35 for IL-1 β pro biological activity. Subunits of IL-1 β pro may be constructed by deleting terminal or internal residues or sequences. The resulting polypeptide should have IL-1 β pro biological activity as defined herein.

The terms "IL-1 β pro", "human IL-1 β pro" or "IL-1 β protease" include, but are not limited to, analogs or subunits of IL-1 β pro which are substantially similar to human IL-1 β pro and/or which exhibit the substrate-specific proteolytic biological activity associated with IL-1 β pro as described herein.

5 The term "substantially similar", when used to describe amino acid sequences, means that a particular sequence may vary from a disclosed reference sequence by one or more substitutions, deletions, or additions. However, the net effect is the same protease biological activity characteristic of the reference human IL-1 β pro polypeptide as determined by a Western gel assay. A substantially similar human IL-1 β pro polypeptide,
10 for example, can have a truncated sequence comprising a "core region" of a sequence of amino acids necessary for the specific protease biological activity characteristic of IL-1 β pro. Substantially similar IL-1 β pro derivatives will be greater than about 30% similar to the corresponding sequence of human IL-1 β pro and have IL-1 β pro biological activity. Polypeptides having amino acid sequences of lesser degrees of similarity but comparable
15 biological activity (including substrate specificity) are considered to be equivalents. More preferably, the derivative polypeptides will have greater than 80% amino acid sequence homology to human IL-1 β pro polypeptide. Percent similarity may be determined, for example, by comparing sequence information using a GAP computer program, version 6.0, available from University of Wisconsin Genetics Computer Group. The GAP
20 program uses the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482 1981). Briefly, the GAP program defines similarity as the number of aligned symbols which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a weighted comparison matrix for
25 amino acids (see, Schwartz and Dayhoff, eds. *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-58 1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

30 "Biologically active", as used herein, refers to IL-1 β pro biological activity to cleave a particular amino acid sequence at the peptide bond between an Asp residue and an Ala or Gly residue.

 "Recombinant", as used herein, means that a polypeptide is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant
35 microbial" defines a polypeptide produced in a microbial expression system which is substantially free of native endogenous substances. Polypeptides expressed in most

bacterial expression systems (e.g., *E. coli*) will be free of glycan. Polypeptides expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

The IL-1 β pro protease has a highly restricted substrate specificity. Human precursor IL-1 β polypeptide has an amino acid sequence His-Asp-Ala-Pro for residues 115-118. Human IL-1 β pro cleaves this sequence between residues 116 and 117 (Asp-Ala) to form human mature IL-1 β polypeptide. Changing Asp-116 to Ala in a human precursor IL-1 β polypeptide by site-directed mutagenesis prevented cleavage of the mutant IL-1 β polypeptide derivative.

Isolated human IL-1 β pro was able to cleave at its specific substrate site even when the tertiary structure of the substrate precursor IL-1 β polypeptide was altered by denaturing the substrate polypeptide in boiling water. Precursor human IL-1 β was denatured by boiling a solution of precursor IL-1 β for fifteen minutes. Denaturation had little effect on the ability of human IL-1 β pro to be able to cleave human precursor IL-1 β into mature IL-1 β . Thus, the tertiary structure of the substrate polypeptide does not significantly contribute to the reaction with the enzyme IL-1 β pro.

IL-1 β pro biological activity was determined by a protease assay. As the IL-1 β pro enzyme is salt sensitive, samples having a salt concentration greater than 50mM were initially desalted. Samples can be desalted, for example, by applying 100 μ l of sample to a pre-spun 1 ml Biogel P-6DG (Bio-Rad) column, which was equilibrated in 10 mM Tris-HCl, 5 mM dithiothreitol, pH 8.1, and centrifuging for 5 min at 1876 X g. The assay was conducted by incubating a mixture of five μ l (30 ng) of purified human IL-1 β precursor and 10 μ l of the sample to be tested for IL-1 β protease biological activity for 60 min at 37°C. A control sample was similarly incubated to check for endogenous IL-1. The control sample mixture contained 5 μ l of 10 mM Tris-HCl, pH 8.1 and 5 mM dithiothreitol instead of IL-1 β precursor. The control sample incubations were terminated by addition of SDS (sodium dodecyl sulfate) in sample buffer followed by five min of boiling.

All incubated assay mixtures were electrophoresed on 0.75 mm-thick SDS, 14% polyacrylamide slab gels, using a discontinuous system, such as the one described in Laemmli *Nature* 277:680 1970. Western blots were performed following electrophoresis by transferring the proteins onto nitrocellulose (Sartorius) and probing using an 20 μ g/ml solution of purified IL-1 β COOH-terminal-specific monoclonal antibody (i.e., 16F5). The blot was developed using Horseradish Peroxidase Color Developing Reagent (Bio Rad). One hundred ng of purified mature IL-1 β was used as a control 17,500 dalton marker on the Western blot.

Human IL-1 β pro enzyme was obtained and purified from THP-1 cells obtainable from the American Type Culture Collection (ATCC). Approximately 120 liters of cells were cultured and then stimulated for 16 hours with liposaccharide, hydroxyurea and silica

as described in Matsushima *Biochemistry* 25:3424 1986. The cells were harvested by centrifugation, washed in Hanks balanced salt solution, and then recentrifuged. The cells were resuspended in 10 mM Tris-HCl, 5 mM dithiothreitol, pH 8.1 at a density of 10^8 /ml. The suspended cells were frozen and thawed three times and the lysates stored at -80°C until further use. Prior to purification, the lysates were thawed and then centrifuged for 20 min at $47,800 \times g$ at 4°C . The supernatant was taken for further purification. The freeze-thawing procedure repeated four times released over 50% of the IL-1 β pro activity into the supernatant. Additional freeze-thaws did not increase the yield of soluble material.

The human IL-1 β pro polypeptide was purified in a six-step process. All the chromatography steps were performed at 4°C using a Pharmacia FPLC System. DEAE-Sephacel, Hydroxyapatite and Blue Agarose gels were pretreated with 0.1% Triton-X-100 and 10% bovine calf serum to prevent non-specific absorption of proteins to the gels. Further, the Blue Agarose column was washed with 8M urea to remove any noncovalently absorbed dye.

1. Approximately 500-600 ml of lysate supernatant was diluted 1:2 in 10mM Tris-HCl and 5mM dithiothreitol, pH 8.1 ("buffer A") to reduce ionic strength of the lysate to $<20\text{mM}$. pH was adjusted to 8.1. The diluted lysate supernatant was applied to a DEAE-Sephacel column (20 x 4.4 cm, Pharmacia Fine Chemicals), equilibrated with buffer A. The flow rate was 120 ml/hr. The column was washed with two column volumes of buffer A and then eluted with a linear gradient (3 column volumes) ranging from 0 to 300 mM NaCl in buffer A. Fifteen ml fractions were collected, analyzed for IL-1 β pro activity and stored for further purification. The IL-1 β pro activity was eluted with between 0.07 and 0.13 M NaCl. This step removed 79% of the contaminating proteins. The bulk of the contaminating proteins eluted between 0.15 and 0.25 M NaCl. This step was further useful in partially removing endogenous mature IL-1 β , which eluted between 0.06 and 0.11 M NaCl, and endogenous precursor IL-1 β which eluted between 0.12 and 0.18 M NaCl.

2. The pooled active fractions from the DEAE column were diluted in 50 mM potassium phosphate buffer, 5 mM dithiothreitol, pH 7.0 ("buffer B"). A 14 x 3 cm column of hydroxyapatite (HA Ultrogel, IBF Biotechnics) was equilibrated with buffer B. The diluted fractions were applied to the equilibrated hydroxyapatite column at a flow rate of 60 ml/hr. The column was washed with 2 column volumes of buffer B and then eluted with a linear gradient (4 column volumes) ranging from 50-200 mM potassium phosphate. Fractions were collected as 10 ml volumes, analyzed for IL-1 β pro activity, and stored for further purification. IL-1 β pro eluted between 0.085 and 0.113 M potassium phosphate. Forty percent of the contaminating polypeptides eluted before the protease and 40% eluted

later than the protease. Further, endogenous mature IL-1 β eluted between 0.05 and 0.08 M potassium phosphate.

3. A 20 x 1.6 cm Blue Agarose column (Gibco-BRL) was equilibrated with buffer A. Fractions from the hydroxyapatite column with activity were diluted 1:3 in buffer A to reduce ionic strength to <30 mM. This was necessary in order to allow IL-1 β pro to bind to the column. Diluted fractions were applied to the Blue Agarose column at a 30 ml/hr rate. The column was washed with three column volumes of buffer A. The proteins were eluted with five column volumes of a linear gradient ranging from 0.1 to 1 M NaCl in buffer A. Ten ml fractions were collected, analyzed for IL-1 β pro activity and stored for further purification. IL-1 β pro was eluted with 0.5 to 0.68 M NaCl. Eighty percent of the contaminating proteins were removed in this step, with 20% eluting earlier and the remaining 60% remaining bound to the column.

4. A 95 x 2.5 cm Sephadex G-75 column (Pharmacia Fine Chemicals) was equilibrated in buffer A and initially calibrated with ferritin (MW 400,000), ovalbumin (MW 43,000), soybean trypsin inhibitor (MW 20,000) and DNP-aspartic acid (MW 300). The Blue Agarose column fractions containing protease activity were pooled and concentrated on a Centriprep-10 concentrator (Amicon) to a volume of approximately 2 ml and then applied to the Sephadex G-75 column. Proteins were eluted with buffer A at a flow rate of 20 ml/hr. Four ml fractions were collected and the fractions containing protease activity were pooled for further purification. IL-1 β pro activity was eluted with between 196 and 220 ml. This position is identical to the elution position of soybean trypsin inhibitor, which suggests that human IL-1 β pro has a molecular weight of about 20,000 daltons. This step removed over 90% of the contaminating proteins from the preparation. Thus, through the Sephadex step, more than 99.8% of the starting protein contaminants have been separated from IL-1 β pro. However, PAGE (polyacrylamide gel electrophoresis) analysis of the fractions still revealed several protein bands that did not correlate with IL-1 β pro biological activity.

5. Fractions from the Sephadex column which contained protease activity were pooled and the pool was concentrated on pretreated Centriprep 10 Concentrators to a 500 μ l volume. Since protein concentration of the Sephadex pool was low (<30 μ g/ml), pretreatment of the centripreps with bovine serum albumin reduced loss of IL-1 β pro activity during concentration. Extensive washing of the treated centripreps prior to use prevented contamination of samples with albumin. Pretreatment was accomplished by centrifuging 15 ml of 1% bovine serum albumin (BSA) in centripreps for 30 min, decanting the remaining solution, and washing with 10 mM Tris-HCl. A Mono P5/20 FPLC chromatofocussing column (Pharmacia Fine Chemicals) was equilibrated with 25 mM Tris-acetate and 5 mM dithiothreitol, pH 8.3 buffer. The concentrated solution was

mixed (1:1 v/v) with 500 µl of 25 mM Tris-acetate and 5 mM dithiothreitol, pH 8.3 and applied to the Mono P5/20 FPLC column. Proteins were eluted with Polybuffer 96:Polybuffer 74 (3:7) pH 5.0 (Pharmacia) at a 15 ml/hr flow rate. One ml fractions were collected and analyzed for pH and biological protease activity. This chromatofocusing step
5 increased the purity of IL-18 pro a further 100 fold and allowed for the visualization of a single protein band that correlated with IL-18 pro biological activity. IL-18 pro was eluted off the chromatofocusing column between pH 6.95 and 6.70. The fractions were concentrated on BSA-pretreated Centricon 10 Concentrators (Amicon) from 1 µl to 50 µl.

6. The fractions were subjected to electrophoresis on a polyacrylamide gel
10 (PAGE), followed by electroblotting onto polyvinyl difluoride membrane paper (PVDF, Millipore Immobilon-P®) at 300 mA for 30 min. The PVDF membrane was stained with Coomassie Blue. There were five major bands with molecular weights of approximately 45,000, 43,000, 36,000, 22,000 and 18,000 daltons. The 22,000 dalton band correlated with IL-18 pro activity and was sequenced.

15 The N-terminal sequence of the 22,000 dalton band yielded an amino acid sequence described herein. A mature human IL-18 pro cDNA or an active fragment thereof was cloned using this N-terminal amino acid sequence and a three-stage polymerase chain reaction (PCR) procedure. In the first stage PCR procedure, fully degenerate PCR primers were designed and made from the N-terminal amino acid sequence. The degenerate
20 primers were used to amplify IL-18 pro-specific sequences from a cDNA library prepared from THP-1 cell mRNA. A random primed first strand THP-1 cDNA library was constructed according to supplier instructions (Amersham). A mixed oligonucleotide primed amplification was carried out according to the procedure described in Lee et al. "cDNA Cloning Using Degenerate Primers" in *PCR Protocols* (Innis, Gelfand, Sninsky and White eds.) Academic Press, Inc. New York pp. 46-53 1990. Primer #1 was
25 designed to cross-hybridize to IL-18 pro DNA (nucleotides 1-17) and contain an *Eco*R1 restriction site. Primer #1 (Sequence ID No. 3) had the sequence:

5'-GTCGAATTCAA(T/C)CCNGCNATGCCNAC-3'

Primer #2 was designed to cross-hybridize to IL-18 pro DNA (complementary to
30 nucleotides 31-47 and contain an *Xba*I restriction site. Primer #2 (Sequence ID No. 4) had the sequence

5'-GTCTCTAGAAG(T/C)TTNAC(A/G)TTNCC(T/C)TC-3'

PCR amplification was performed with *thermus aquaticus* polymerase (Perkin-Elmer Cetus) in 100µl of buffer for 30 cycles as described in Lee et al. *infra*. A 63 bp amplified
35 fragment was obtained from PCR amplification. This amplified fragment was subcloned into a pGem-4 vector (Promega). DNA sequence analysis of 10 isolates indicated that this

fragment encoded the first 16 amino acids of the N-terminus of IL-18 pro as determined by purification and N-terminal sequence analysis.

The second stage of the PCR procedure made Primer #3 composed of nucleotides 1-17 (Figure 1) and a *Not*I restriction site and Primer #4 containing 20 T residues and a *Not*I restriction site. Primers #3 and #4 were added to the THP-1 cDNA library described above and PCR amplified for 6 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and for 24 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Southern analysis of the PCR amplified clone using a 17 base oligonucleotide probe (complementary to nucleotides 16-32 in Figure 1) found a band at approximately 1000 bp that was also found to possess IL-18 pro biological activity. The 1000 bp DNA was gel purified, subjected to a similar second round of PCR and subcloned into pGem-5 for sequencing. The nucleotide sequence of this clone is shown in Figure 1.

In the third stage of PCR cloning, full length IL-18 pro clones were isolated from a cDNA library prepared from peripheral blood neutrophils. We found that neutrophils expressed IL-18 pro mRNA. We isolated two clones (p48 and p214) with IL-18 pro specific inserts of 1367 and 1360 base pairs, respectively. The DNA sequence shown in Figure 1 is a composite of all the IL-18 pro clones. The amino acids encoded by all of the IL-18 pro clones we found were identical.

IL-18 pro cDNA is approximately 1373 base pairs in length, including a stretch of A nucleotides corresponding to the poly (A) tail of mRNA. These A residues are preceded by two polyadenylation signals, AATAA, at 1316 and 1335 base pair. The sequence has an open reading frame of 404 amino acids, starting with an initiator Met codon at nucleotide 18 and ending with a termination codon at nucleotide 1230. Initiation of translation could also begin with an in-frame Met codon at nucleotide 66. Both initiator Met codons have consensus Kozak translation initiation sequences. Polypeptides initiated with the Met residue at position 51 also have biological activity.

IL-18 pro is a cytoplasmic enzyme. As the purified enzyme N-terminal amino acid is Asn (120), the protease undergoes N-terminal processing resulting in removal of 119 amino acids or 69 amino acids if the alternate initiator codon is used. Deletion analysis has indicated that at least 107 amino acids are removed from the C-terminus. However, it appears that the full C-terminus is necessary for proper folding of the protease before approximately 107 C-terminal amino acids can be removed to insure biological activity for the protease.

Expression of Recombinant IL-18 pro

The DNA sequence shown in Figure 1 was expressed in a mammalian cell (e.g., COS-7 cells). For mammalian cell expression, synthetic oligonucleotide primers were made to amplify the entire coding domain of IL-18 pro. The 5' primer (5'-

ATATCGGTACCGCCTCCAGCATGCCTCCGGCAATGCCCACATC-3') contains an *Asp*718 restriction site and an initiator Met residue fused to the N-terminus of the enzyme (nucleotides 1-20). The 3' primer (5'-CTGCTAGATCTGCCCGCAGACATTTCATACAG-3') contains a *Bgl*2 restriction site and is complementary to nucleotides 883-902 of Figure 1. The PCR generated fragment was ligated into pDC303 mammalian vector, as described in Mosley et al. *Cell* 59:335-348 1989.

Human IL-1 β pro is preferably produced by recombinant DNA techniques. A recombinant DNA expression system inserts a clone encoding human IL-1 β pro polypeptide or a derivative thereof with biological activity into an expression vector. The expression vector is inserted into a host cell. The host cell's protein synthesis machinery synthesizes the recombinant human IL-1 β pro polypeptide.

Suitable host cells for expression of mammalian IL-1 β pro polypeptides or derivatives thereof include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Suitable prokaryotic hosts cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce mammalian IL-1 β pro polypeptides or derivatives thereof using RNAs derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985.

When an IL-1 β pro polypeptide or derivative thereof is expressed in a yeast host cell, the nucleotide sequence (e.g., structural gene) that codes on expression for an IL-1 β pro polypeptide or derivative thereof may include a leader sequence. The leader sequence enables improved extracellular secretion of translated polypeptide by a yeast host cell.

Alternatively, in a prokaryotic host cell, such as *E. coli*, the IL-1 β pro polypeptide or derivative thereof may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant IL-1 β pro polypeptide or derivative thereof. Moreover, prokaryotic host cells may be used for expression of IL-1 β pro polypeptides or derivatives thereof that do not require extensive proteolytic and disulfide processing.

The recombinant expression vectors carrying the recombinant IL-1 β pro structural gene nucleotide sequence or derivative thereof are transfected or transformed into a substantially homogeneous culture of a suitable host microorganism or mammalian cell

line. Examples of suitable host cells include bacteria such as *E. coli*, yeast such as *S. cerevisiae*, or a mammalian cell line such as Chinese Hamster Ovary (CHO) cells.

Transformed host cells are cells which have been transformed or transfected with IL-1 β pro or a derivative thereof structural gene nucleotide sequences. Expressed IL-1 β pro polypeptides will be located within the host cell and/or secreted into culture supernatant, depending upon the nature of the host cell and the gene construct inserted into the host cell. Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers. A phenotypic selectable marker is, for example, a gene encoding proteins that confer antibiotic resistance or that supply an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Other useful expression vectors for prokaryotic host cells include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and an IL-1 β pro structural gene sequence. Other commercially vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences are commonly used for recombinant prokaryotic host cell expression vectors. Common promoter sequences include β -lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

Human IL-1 β pro polypeptides and derivative polypeptides may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2 μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, and sequences for transcription termination. Preferably, yeast vectors include an origin of replication sequence and selectable marker. Suitable promoter sequences for yeast vectors include promoters for metallothionein, 3-phosphoglycerate

kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate
5 mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP-A-73,657.

Yeast vectors can be assembled, for example, using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication). Other yeast
10 DNA sequences that can be included in a yeast expression construct include a glucose-repressible ADH2 promoter and α -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast α -factor leader sequence directs secretion of heterologous polypeptides. The α -factor leader sequence is often inserted between the promoter
15 sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such
20 protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp⁺ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μ g/ml adenine and 20 μ g/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may
25 be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μ g/ml adenine and 80 μ g/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express
30 recombinant IL-1 β pro polypeptide or derivatives thereof. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells (Gluzman *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells, Chinese hamster ovary (CHO) cells, HeLa cells, and BHK cell lines. Suitable mammalian expression vectors include nontranscribed elements such as an origin of replication, a promoter sequence, an enhancer linked to the
35 structural gene, other 5' or 3' flanking nontranscribed sequences, such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Transcriptional and translational control sequences in mammalian host cell expression vectors may be provided by viral sources. For example, commonly used mammalian cell promoter sequences and enhancer sequences are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Further, mammalian genomic IL-1 β pro promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983).

Purification of Recombinant Human IL-1 β pro

Purified human IL-1 β pro polypeptides or derivatives thereof are prepared by culturing transformed host cells under culture conditions necessary to express IL-1 β pro polypeptides or derivatives thereof. The expressed polypeptides are purified from culture media or cell extracts. For example, supernatants from cultured transformed host cells can secrete recombinant IL-1 β pro polypeptide into culture media. The IL-1 β pro polypeptide or derivative thereof is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix. For example, a suitable purification matrix is an IL-1 β pro inhibitor or an antibody molecule specific for an IL-1 β pro polypeptide or derivative thereof and bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify an IL-1 β pro polypeptide composition. Some or all of the foregoing purification steps, in various

combinations, can also be employed to provide a homogeneous recombinant protein. Alternatively, some or all of the steps used in the purification procedure described herein can also be employed.

5 Recombinant polypeptide produced in bacterial culture is usually isolated by initial disruption of the host cells, extraction from cell pellets if an insoluble polypeptide, or from the supernatant if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange or size exclusion chromatography steps. Finally, reverse phase high performance liquid chromatography (RP-HPLC) can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw
10 cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells will generally express IL-1 β pro polypeptide as a secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential,
15 reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Administration of IL-1 β pro Polypeptide and Derivative Compositions

The present invention provides methods of using therapeutic compositions comprising an effective amount of IL-1 β pro polypeptides and derivatives thereof in a
20 suitable diluent and carrier. For therapeutic use, purified IL-1 β pro or a biologically active derivative thereof is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, IL-1 β pro compositions administered to suppress autoimmunity can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, an IL-1 β
25 pro therapeutic agent will be administered in the form of a pharmaceutical composition comprising purified polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining IL-1 β pro with buffers, antioxidants such as ascorbic acid, low molecular
30 weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

The pharmaceutical compositions of the present invention include one or more of the IL-
35 1 β pro inhibitor compounds of this invention formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively

referred to herein as carriers, for parenteral injection, for oral administration in solid or liquid form, for rectal or topical administration, and the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenously, intramuscularly or subcutaneously), intracisternally, intravaginally, 5 intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols 10 (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, 15 emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for 20 example, aluminum monostearate and gelatin.

If desired, and for more effective distribution, the compounds can be incorporated into slow release or targeted delivery systems such as polymer matrices, liposomes, and microspheres. They may be sterilized, for example, by filtration through a bacteria-retaining 25 filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) 30 binders, as for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for 35 example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid

polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

5 Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like.

Solid dosage forms such as tablets, dragees, capsules, pills and granules can be prepared with coatings and shells, such as enteric coatings and others well known in this art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner.

10 Examples of embedding compositions which can be used are polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the
15 liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butylene glycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol,
20 polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as
25 for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating
30 excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore, melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile
35 conditions with a physiologically acceptable carrier and any needed preservatives, buffers or propellants as may be required. Ophthalmic formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

The compounds of the present invention can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to the IL-18 pro inhibiting compounds of the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic.

Methods to form liposomes are known in the art. See, for example, Methods in Cell Biology, Ed. by Prescott, Volume XIV, Academic Press, New York, N.Y., p. 33 et seq., (1976).

Actual dosage levels of active ingredient in the compositions of the present invention may be varied so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

The total daily dose of the compounds of this invention administered to a host in single or divided doses may be in amounts, for example, of from about 0.1 mg to about 160.0 mg per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

The following examples are for the purposes of illustration and not by way of limitation.

EXAMPLE 1

This example illustrates the range of substrate specificity of purified human IL-18 pro enzyme to cleave a group of amino acid sequences. A variety of peptide substrates were prepared featuring changes in individual amino acids in the region corresponding to the cleavage site in human precursor IL-18 (His115 to Pro118). The reactivity of the peptide substrates was expressed relative to the peptide corresponding to Ala112 to Ser121 of the precursor IL-18 sequence.

Substrate peptides were synthesized by solid phase method (Merrifield *J. Amer. Chem. Soc.* 86:304-05 1964) using either an Applied Biosystems 430A peptide synthesizer or by the manual T-bag approach of Houghten (*Proc. Nat. Acad. Sci. USA* 82:5131-35

1985). 4-Methyl benzhydrylamine resin was used. The substrate peptides were acetylated prior to cleavage from resin, by liquid HF (0°C, 1 hr) in the presence of anisole as scavenger (HF:anisole 9:1). After evaporation of HF, the substrate peptide resin mixtures were washed with diethyl ether and extracted with 15% (w/v) acetic acid, lyophilized and
5 purified on reverse phase high performance liquid chromatography (RP-HPLC) on a Vydac C18, 2.2cm x 25 cm column. Trifluoroacetic acid (0.1%) in water was solvent A and 0.1% trifluoroacetic acid in acetonitrile was solvent B for the mobile phases.

The purified substrate peptides were characterized by amino acid analysis using a Beckman 6300 system, RP-HPLC and mass spectrometry. Mass spectra were obtained by
10 either fast atom bombardment on a VG Trio-2 system with xenon as the ionizing gas and glycerol/thioglycerol (1:1) as the sample matrix or by ²⁵²Cf plasma desorption mass spectrometry on a Bio-Ion 20 mass spectrometer (See Tsarbopoulos *Peptide Res.* 2:258-66 1989). In each case, the mass of the observed peptide substrate corresponded with the theoretical value.

15 Peptide solutions of standard concentration were prepared by dissolving about 2-3 mg of peptide substrate in water, loading the solution onto a Waters Sep-Pak C18 cartridge and washing three times with 5 ml of water. The peptide substrates were eluted with acetonitrile and then evaporated to dryness. Each substrate was standardized to 1 mM by amino acid analysis prior to use.

20 Purified human IL-18 pro enzyme (10μl), peptide substrate in water (10μl), and 10 mM Tris buffer, pH 8.0 containing 25% v/v glycerol (10μl) were mixed and the mixtures were incubated at 37°C for four hours. The reaction was quenched with by adding 1 M glycine/HCl buffer pH 2.0 (10μl). The samples were then analyzed using RP-HPLC with a Vydac C18 column (0.46 cm x 25 cm) and eluting with a linear gradient from 100%
25 solvent A to 70% solvent A/30% solvent B over 30 min at a flow rate of 1 ml/min. The effluent was monitored at 280 nm except with digestions of Ac-VHDAPV-NH₂ and Ac-HDAP-NH₂ (see Example 2) which were monitored at 220 nm. In the case of substrate peptides cleaved by the protease, consumption of substrate peptide was observed together with formation of a 280 nm absorbing product. A comparison of peak area of product
30 peptide to that of total peak area of substrate and product yielded the extent of peptide cleavage, because the area under the combined substrate and product peaks was constant and independent of the amount of cleavage by the IL-18 pro enzyme. Identities of peptide product peaks were confirmed by amino acid analysis and by mass spectrometry.

Table 1 shows the relative reactivities of a series of eight peptide substrates that
35 were subject to digestion by purified IL-18 pro enzyme.

TABLE 1

<u>Peptide</u>	<u>Sequence</u>	Reactivity relative to peptide 1
5		
1	AYVHDAPVRS	1.00
2	AYVHNAPVRS	<0.01
10		
3	AYVHEAPVRS	<0.05
4	AYVH(D-Asp)APVRS	<0.01
5	AYVHDGPVRS	3.40
15		
6	AYVHDVPVRS	<0.05
7	AYVFDAPVRS	0.50
20		
8	AYVHDAAVRS	0.47

The cleavage site can be described with the corresponding human precursor IL-1 β amino acid residues as follows:

P2 P1 P1' P2'

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His-Asp-Ala-Pro

Changing the L-aspartic acid residue of peptide 1 to either asparagine (peptide 2), glutamic acid (peptide 3) or D-aspartate (peptide 4) has a profound effect on the ability of IL-1 β pro to cleave the substrate. These data establish the requirement of an L-aspartate residue in the P1 position for this enzyme to be able to cleave a substrate.

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Peptides 5 and 6 represent changes in the P1' position of the human precursor IL-1 β cleavage site. Replacing alanine with glycine (peptide 5) results in a substrate that is 3.4 times more reactive than peptide 1. However, changing the same residue to a valine (peptide 6) effectively prevents proteolytic cleavage. The fact that with a glycine residue in P1' the peptide is cleaved more readily suggests that the alanine residue in human precursor IL-1 β polypeptide is not critical for substrate binding, while the result with a valine residue in the P1' position indicates low steric tolerance at the P1' position. Thus, it seems

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unlikely that the IL-1 β pro enzyme or derivatives thereof will cleave anywhere other than between Asp-Gly and Asp-Ala residues.

Peptides 7 and 8 represent changes to the P2 and P2' sites, respectively. Changing the proline of peptide 1 to an alanine yielded a substrate which was still cleaved by human IL-1 β pro but only half as efficiently as the peptide with human IL-1 β native sequence. A similar result was obtained when the histidine of peptide 1 was replaced with a phenylalanine. These data suggest that human IL-1 β pro enzyme tolerates conservative replacements of both residues and that the P2 and P2' positions are not as vital for activity as the amino acids at the P1 and P1' positions.

10

EXAMPLE 2

This example illustrates the effect of substrate peptide length on the ability of human IL-1 β pro enzyme to cleave peptide substrates. The experiment was conducted as described in Example 1. Five substrate peptides were made that correspond to the amino acid sequence of the IL-1 β pro cleavage site of human precursor IL-1 β . The results are shown in Table 2 below:

Table 2		
<u>Peptide</u>	<u>Sequence</u>	Reactivity relative <u>to peptide 1</u>
1	AYVHDAPVRS	1.00
9	EAYVHDAPVRSL	0.74
10	YVHDAPVR	2.40
11	VHDAPV	Not cleaved
12	HDAP	Not cleaved

The eight amino acid peptide (Ac-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-NH₂) is cleaved most efficiently while the four and six amino acid peptides are not cleaved. Thus, IL-1 β pro has a minimum number of amino acid residues necessary for substrate peptide cleavage.

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EXAMPLE 3

Synthesis of IL-18 Protease InhibitorsA. Synthesis of Boc-Asp-CH₂F.

5 A suspension of Boc-Asp-OH (8.11 mmol) and fluoroacetic anhydride (16.2 mmol) in benzene (30 ml) was treated with triethylamine (16.2 mol) at room temperature. The catalyst 4-dimethylaminopyridine (0.41 mmol) was added to the solution and the reaction stirred for about 2 h at room temperature. About 100 ml benzene was added to the reaction mixture. The organic solution was washed with 1N HCl (2 X 50 ml), saturated NaHCO₃ (2 X 50 ml), and 10 saturated NaCl (2 X 50 ml), followed by drying over anhydrous MgSO₄. The solvent was then removed by evaporation under reduced pressure. The resulting oil was applied to a 2.5 X 80 cm column of silica gel (60-200 mesh). The title compound was eluted with 2% methanol in chloroform.

15 B. Synthesis of Boc-His-Asp-CH₂F, Boc-Pro-Asp-CH₂F, Boc-Tyr-Asp-CH₂F and Boc-Phe-Asp-CH₂F.

Boc-Asp-CH₂F prepared in accordance with the method of Example 3A above may be dissolved in trifluoroacetic acid (TFA) and the mixture stirred for about 5 minutes at about 23°C. Cold ether may then be added to the mixture. The ether is evaporated and toluene added 20 to co-evaporate residual TFA. The deprotected peptide (H-Asp-CH₂F) is obtained as a TFA salt. The deprotected peptide may then be coupled to a protected amino acid (i.e. Boc-HisOH, Boc-ProOH, Boc-TyrOH, Boc-PheOH) using a standard symmetric anhydride procedure employing dicyclohexylcarbodiimide as a coupling reagent. Bodanszky, *supra*.

25 C. Synthesis of Ac-His-Asp-CH₂F, Ac-Pro-Asp-CH₂F, Ac-Tyr-Asp-CH₂F and Ac-Phe-Asp-CH₂F.

The Boc protecting group may be removed from the compounds made in accordance with the method of Example 3B using trifluoroacetic acid as described above. Each deprotected compound may then be acetylated with acetic anhydride and diisopropylamine (DIAE) 30 according to standard techniques. Stuart et al. *supra*.

D. Synthesis of Cbz-His-Asp-CH₂F, Cbz-Pro-Asp-CH₂F, Cbz-Tyr-Asp-CH₂F and Cbz-Phe-Asp-CH₂F.

35 The Boc protecting group may be removed from Boc-Asp-CH₂F prepared according to the method of Example 3A using TFA as described above. Benzyloxycarbonyl-protected amino acids (i.e. Cbz-His-OH, Cbz-Phe-OH, Cbz-Tyr-OH, Cbz-Pro-OH) available from commercial

sources (Bachem, Philadelphia, PA) can then be coupled to the deprotected Asp using a symmetric anhydride coupling procedure. Bodanszky, *supra*.

EXAMPLE 4

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Inhibition of IL-1 β pro Activity

Boc-Asp-CH₂F was tested for its ability to inhibit IL-1 β pro catalyzed degradation of precursor IL-1 β using an *in vitro* assay method. Black et al. I. Boc-Asp-CH₂F was prepared in accordance with the method of Example 3A.

10 5 μ l of precursor IL-1 β (about 50 μ g/ml in PBS) was mixed with 10 μ l of purified IL-1 β pro (15-75 μ g/ml in PBS) and incubated at 37°C for 30 min. The incubation was terminated by placing the samples on dry ice or by the addition of SDS sample buffer. PMSF (phenylmethanesulfonyl) was then added to a concentration of 1 mM, and the samples were dialyzed against water. After dialysis, the samples were concentrated to dryness in a Speed-Vac concentrator and dissolved in SDS sample buffer.

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SDS-PAGE was carried out with 12% polyacrylamide gels. The gels were placed in transfer buffer (0.192 M glycine, 0.025 M Tris-HCl (pH 8.3), 20% v/v methanol), and protein was then electrophoresed onto nitrocellulose (Sartorius) in a Hoeffer transfer apparatus (1 h at maximum voltage). The nitrocellulose was subsequently placed in 20 mM sodium phosphate, pH 7.4 (PBS) containing 3% bovine serum albumin for at least 15 min at room temperature.

20

We used a monoclonal antibody (MAb) specific for mature IL-1 β to probe the blot. MAb was added to a concentration of 9 μ g/ml, and the incubation was continued for 30 min. The blot was then rinsed three times with PBS and was developed with a solution obtained by mixing 6 mg of horseradish peroxidase developing reagent (Bio-Rad) dissolved in 2 ml methanol and hydrogen peroxide (60 μ l diluted into 10 ml of Tris-buffered saline).

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The data show that Boc-Asp-CH₂F completely inhibits the generation of mature IL-1 β from precursor IL-1 β at a concentration of 5 μ M and partially inhibits generation of mature IL-1 β at a concentration of 1 μ M.

EXAMPLE 5

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This example illustrates biological activity of IL-1 β pro when transfected into COS cells. We inserted a cDNA corresponding to amino acids 120 to 404 into a mammalian cell expression vector (pDC303). This plasmid was co-transfected into COS-7 cells (monkey kidney) with a second mammalian expression plasmid containing a cDNA encoding precursor IL-1 β . After two days, cells were radiolabeled with ³⁵S and IL-1 β specific
35 proteins were immunoprecipitated from cell lysates. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. We found that transfected COS-7 cells can process precursor IL-1 β to mature IL-1 β only if the cells were co-transfected with a

plasmid encoding IL-18 pro. Cells co-transfected with a control plasmid or cells mock transfected did not show any processing of precursor IL-18. Thus, IL-18 pro, lacking the N-terminal 119 amino acids enables cells to process precursor IL-18 to the mature form of this protein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BLACK, ROY
SLEATH, PAUL
KRONHEIM, SHIRLEY

(ii) TITLE OF INVENTION: INTERLEUKIN 1 B PROTEASE

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 0401WO

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: INTERLEUKIN-1B

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ala Glu Val Pro Glu Leu Ala Ser Glu Met Met Ala Tyr Tyr Ser
 1 5 10 15
 Gly Asn Glu Asp Asp Leu Phe Phe Glu Ala Asp Gly Pro Lys Gln Met
 20 25 30
 Lys Cys Ser Phe Gln Asp Leu Asp Leu Cys Pro Leu Asp Gly Gly Ile
 35 40 45
 Gln Leu Arg Ile Ser Asp His His Tyr Ser Lys Gly Phe Arg Gln Ala
 50 55 60
 Ala Ser Val Val Val Ala Met Asp Lys Leu Arg Lys Met Leu Val Pro
 65 70 75 80
 Cys Pro Gln Thr Phe Gln Glu Asn Asp Leu Ser Thr Phe Phe Pro Phe
 85 90 95
 Ile Phe Glu Glu Glu Pro Ile Phe Phe Asp Thr Trp Asp Asn Glu Ala
 100 105 110
 Tyr Val His Asp Ala Pro Val Arg Ser Leu Asn Cys Thr Leu Arg Asp
 115 120 125
 Ser Gln Gln Lys Ser Leu Val Met Ser Gly Pro Tyr Glu Leu Lys Ala
 130 135 140
 Leu His Leu Gln Gly Gln Asp Met Glu Gln Gln Val Val Phe Ser Met
 145 150 155 160
 Ser Phe Val Gln Gly Glu Glu Ser Asn Asp Lys Ile Pro Val Ala Leu
 165 170 175
 Gly Leu Lys Glu Lys Asn Leu Tyr Leu Ser Cys Val Leu Lys Asp Asp
 180 185 190
 Lys Pro Thr Leu Gln Leu Glu Ser Val Asp Pro Lys Asn Tyr Pro Lys
 195 200 205
 Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu Ile Asn Asn
 210 215 220
 Lys Leu Glu Phe Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile Ser Thr
 225 230 235 240
 Ser Gln Ala Glu Asn Met Pro Val Phe Leu Gly Gly Thr Lys Gly Gly
 245 250 255
 Gln Asp Ile Thr Asp Phe Thr Met Gln Phe Val Ser Ser
 260 265

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1659 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: INTERLEUKIN 1B PROTEASE

(vii) IMMEDIATE SOURCE:

(B) CLONE: INTERLEUKIN 1B PROTEASE

(x) PUBLICATION INFORMATION:

(A) AUTHORS: BLACK , ROY
SLEATH, PAUL
KRONHEIM, SHIRLEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAAAGGAGAG AAAAGCCTAA AAGAGAGTGG GTAGATGGCC GACAAGGTCC TGAAGGAGAA	60
GAGAAAGCTG TTTATCCGTT CCATGGGTGA AGGTACAATA AATGGCTTAA GGTAGAAGGT	120
GAAGCAAATA CTGCATGAAT TATTACAGAC AAGGGTGTCTG AACAAGGAAG AGATGGAGAA	180
AGTAAACCT GAAATGCTA CAGTTTATAG AAAAGAAGAA CGCTTATGGA TAAGACCCGA	240
GCTTTGATTG ACTCCGTTAT TCCGAAAGGG GCACAGGCAT GCCAAATTG CATCACATAC	300
CGGATAAGTC AAAGTGATAA TTTGTGAAGA AGACAGTTAC CTGGCAGGGA CGCTGGGACT	360
CTCAGCAGAT CAAACATCTG GAAATTACCT TAATTGAGGA AAGAAAGAA ATTATGCAAG	420
ACTCTCAAGG AGTACTTTCT TCCTTTCCAG CTCCTCAGGC AGTGCAGGAC AACCAGCTA	480
TGCCCACAGG GAACGGAAGA GTGAATCCTC AGGCTCAGAA GGAATGTCA AGCTTTGCTC	540
CCTAGAAGAA GCTCAAAGGA TATGGAAACA AAAGTCGGCA GTTAAGTAGA ACAGGAGAGA	600
TTTATCCAAT AATGGACAAG TCAAGCCGCA CACGTCTTGC TCTCATTATC TGCAATGAAG	660
AATTTGACAG TAGAGTGAAG AATGTTTGTAG TAATTCCTAG AAGAACTGGA GCTGAGGTTG	720
ACATCACAGG CATGACAATG CTGCTACAAA ATCTGGGGTA CAGCGTAAA TAAATTTGGA	780
AAAAGGGATG TGAAAAAAA TCTCACTGCT TCGGACATGA CTACAGAGCT GGAGGCATTT	840
GCACACCGCC CAGAGCACAA GTATATGAGG GCGGACCTCT GACAGCACGT TCCTGGTGTT	900
CATGTCTCAT GGTATTCGGG AAGGCATTTG TGGGAAGAAA CACTCTGAGG AAGAAAATAT	960
ACACAAGTCC CAGATATACT ACAACTCAAT GCAATCTTTA ACATGTTGAA TACCAAGAAG	1020
TGCCCAGTT TGAAGGACAG AACAGGAGAA TAAGAAACCG AAGGTGATCA TCATCCAGGC	1080
CTGCCGTGGT GACAGCCCTG GTGTGGTGTG GTTTAAAGAT TCAGTAGGAA GATTGGGAAA	1140
AAAGGTTTCT GGAAACCTAT CTTTACCAAC TACAGAAGAG TTTGAGGATG ATGCTATTAA	1200
GAAAGCCAC ATAGAGAAGA AACTAAATAG TTGAGATTTT ATCGCTTTCT GCTCTTCCAC	1260
ACCAGATAAT GTTTCTTGGA GACATCCCAC AATGGGCTCT GTTTTTATTG AGGTGGTAAC	1320
CAAGGAGAAG GGAAGACTCA TTGAACATAT GCAAGAATAT GCCTGTTCTT GTGATGTGGA	1380

GGAAATTTTC CGCAAGGTTT GATTGGAGA GAAGTTTGAG ATTAGCTTCA TTTGAGCAGC 1440
CAGATGGTAG AGCGCAGATG CCCACCACTG AAAGAGTGAC TTTGACAAGA TGTTTCTACC 1500
TCGTTCCAG GACATTAAAA TAAGGAACT GTATGAATGT CTGCGGGCAG GAAGTGAAGA 1560
GATCGTTCTG TAAAAGGTTT TTGGAATTAT GTCTGCTGAA TAATAACTT TTTTGAAT 1620
AATAAATCTG GTAGAAAAAT GAAAAAAAAA AAAAAAAAAA 1659

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: PRIMER #1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGGAATTCA AYCCNGCNAT GCCNAC

26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: PRIMER #2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTCTCTAGAA GYTTNACRTT NCCYTC

26

WHAT IS CLAIMED IS:

1. An isolated polypeptide having protease activity for a specific protease cleavage site, wherein the protease activity is specific for a substrate peptide having an amino acid sequence comprising:
R₁ - Asp - R₂ - R₃
wherein R₁ and R₃ are independently any D or L isomer amino acid, R₂ is Ala or Gly, and wherein the specific protease cleavage site is between Asp and R₂.
2. The isolated polypeptide of claim 1 wherein the substrate peptide is at least eight amino acids in length.
3. An isolated IL-1 β pro polypeptide having IL-1 β pro activity and encoded by a DNA sequence is selected from the group consisting of the nucleotide sequences in Figure 1 beginning at nucleotide 1 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to a nucleotide from about 851 to about 962, DNA sequences which detectably hybridize to the Figure 1 sequence from nucleotide 1 to nucleotide 1232 and encode a polypeptide displaying biological activity to proteolytically cleave human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues, and DNA sequences which, due to degeneracy of the genetic code, encode a mammalian IL-1 β pro polypeptide encoded by any of the foregoing DNA inserts and sequences.
4. The isolated polypeptide of claim 3 wherein the protein sequence is selected from the group consisting of amino acid 1 to amino acid 404, amino acid 51 to amino acid 404, amino acid 120 to an amino acid beginning at position 278 and extending to amino acid 315, and amino acid 120 to amino acid 404.
5. An isolated DNA sequence encoding a mammalian IL-1 β pro enzyme.
6. The isolated DNA sequence of claim 5 wherein the mammalian IL-1 β pro enzyme is a human IL-1 β pro enzyme.
7. The isolated DNA sequence of claim 5 wherein the DNA sequence is selected from the group consisting of the nucleotide sequences in Figure 1 beginning at nucleotide 1 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to nucleotide 1232, beginning at nucleotide 374 and extending to a nucleotide from about

- 851 to about 962, DNA sequences which detectably hybridize to the Figure 1 sequence from nucleotide 1 to nucleotide 1232 and encode a polypeptide displaying biological activity to proteolytically cleave human precursor IL-1 β polypeptide at a cleavage site between the Asp 116 and Ala 117 residues, and DNA sequences which, due to degeneracy of the genetic code, encode a mammalian IL-1 β pro polypeptide encoded by any of the foregoing DNA inserts and sequences.
- 5
8. A recombinant expression vector comprising a DNA sequence according to claim 5.
- 10
9. A recombinant expression vector comprising a DNA sequence according to claim 6.
- 15
10. A recombinant expression vector comprising a DNA sequence according to claim 7.
- 20
11. A process for preparing a mammalian IL-1 β pro enzyme or an analog or derivative thereof, comprising culturing a suitable host cell comprising a vector according to claim 8 under conditions promoting expression.
- 25
12. A process for preparing a mammalian IL-1 β pro enzyme or an analog or derivative thereof, comprising culturing a suitable host cell comprising a vector according to claim 9 under conditions promoting expression.
- 30
13. A process for preparing a mammalian IL-1 β pro enzyme or an analog or derivative thereof, comprising culturing a suitable host cell comprising a vector according to claim 10 under conditions promoting expression.
- 35
14. An inhibitor compound comprising:
Z-Q₂-Asp-Q₁
where Z is an N-terminal protecting group; Q₂ is 0 to 4 amino acids such that the sequence Q₂-Asp substantially corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp and Q₁ is an electronegative leaving group.
15. The inhibitor compound according to claim 14 wherein Z is C₁-C₆ alkyl, benzyl, acetyl, C₁-C₆ alkoxycarbonyl, benzyloxycarbonyl or C₁-C₆ alkyl carbonyl.

16. The inhibitor compound according to claim 14 wherein Z is t-butoxycarbonyl, acetyl or benzyloxycarbonyl.
17. The inhibitor compound according to claim 14 wherein Q₁ is an aldehyde, a
5 diazomethyl ketone or a halomethyl ketone.
18. The inhibitor compound according to claim 14 wherein Q₁ is fluoromethyl ketone.
19. The inhibitor compound according to claim 14 wherein Q₂ is 1 amino acid
10 residue.
20. The inhibitor compound according to claim 14 wherein Q₂ is His, Phe, Pro or Tyr.
21. A pharmaceutical composition comprising a physiologically acceptable carrier and an inhibitor compound of the formula:
15
$$Z-Q_2-Asp-Q_1$$
where Z is an N-terminal protecting group; Q₂ is 0 to 4 amino acids such that Q₂-Asp
20 substantially corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp and Q₁ is an electronegative leaving group.
22. A compound selected from the group consisting of Boc-Asp-CH₂F, Boc-His-Asp-CH₂F, Boc-Phe-Asp-CH₂F, Boc-Pro-Asp-CH₂F, Boc-Tyr-Asp-CH₂F, Ac-His-
25 Asp-CH₂F, Ac-Phe-Asp-CH₂F, Ac-Pro-Asp-CH₂F, Ac-Tyr-Asp-CH₂F, Cbz-His-Asp-CH₂F, Cbz-Phe-Asp-CH₂F, Cbz-Pro-Asp-CH₂F and Cbz-Tyr-Asp-CH₂F wherein Boc is t-butoxycarbonyl, Ac is acetyl and Cbz is benzyloxycarbonyl.
23. A method for improving wound healing at a wound site comprising
30 administering a pharmaceutical composition to the wound site comprising the isolated polypeptide of claim 1 in a suitable pharmaceutical carrier.
24. A method for treating arthritis comprising administering a pharmaceutical composition comprising the isolated polypeptide of claim 1 in a suitable pharmaceutical
35 carrier.

25. A method for treating an autoimmune disease in a susceptible individual comprising administering a pharmaceutical composition comprising the isolated polypeptide of claim 1 in a suitable pharmaceutical carrier.

5 26. The method of claim 25 wherein the autoimmune disease is selected from the group consisting of Insulin-dependent diabetes melitus, Graves' disease, Hashimotos disease and a lupus disease.

10 27. A method for reducing the detrimental side effects of radiation treatment comprising administering a pharmaceutical composition comprising the isolated polypeptide of claim 1 in a suitable pharmaceutical carrier.

15 28. A method of inhibiting IL-1 β pro activity in a mammal in need of such treatment comprising administering to said mammal an effective inhibitory amount of an inhibitor compound according to claim 14.

20 29. A method of treating inflammation or preventing and treating an autoimmune disease in a mammal in need of such treatment comprising administering to said mammal an effective amount of an inhibitor compound according to claim 14.


30. An antisense oligonucleotide comprising a sequence of at least 15 nucleotides complementary to a sequence of IL-1 β pro cDNA, wherein said antisense oligonucleotide inhibits translation of IL-1 β pro mRNA.

FIGURE 1

5'—AAAAGGAGAGAAAAGCC																								17	
Met	Ala	Asp	Lys	Val	Leu	Lys	Glu	Lys	Arg	Lys	Leu	Phe	Ile	Arg	Ser	Met	Gly	Glu	Gly	Thr	Ile	Asn	Gly	Leu	25
ATG	GCC	GAC	AAG	GTC	CTG	AAG	GAG	AAG	AGA	AAG	CTG	TTT	ATC	CGT	TCC	ATG	GGT	GAA	GGT	ACA	ATA	AAT	GGC	TTA	92
Leu	Asp	Glu	Leu	Leu	Gln	Thr	Arg	Val	Leu	Asn	Lys	Glu	Glu	Met	Glu	Lys	Val	Lys	Arg	Glu	Asn	Ala	Thr	Val	50
CTG	GAT	GAA	TTA	TTA	CAG	ACA	AGG	GTG	CTG	AAC	AAG	GAA	GAG	ATG	GAG	AAA	GTA	AAA	CGT	GAA	AAT	GCT	ACA	GTT	167
Met	Asp	Lys	Thr	Arg	Ala	Leu	Ile	Asp	Ser	Val	Ile	Pro	Lys	Gly	Ala	Gln	Ala	Cys	Gln	Ile	Cys	Ile	Thr	Tyr	75
ATG	GAT	AAG	ACC	CGA	GCT	TTG	ATT	GAC	TCC	GTT	ATT	CCG	AAA	GGG	GCA	CAG	GCA	TGC	CAA	ATT	TGC	ATC	ACA	TAC	242
Ile	Cys	Glu	Glu	Asp	Ser	Tyr	Leu	Ala	Gly	Thr	Leu	Gly	Leu	Ser	Ala	Asp	Gln	Thr	Ser	Gly	Asn	Tyr	Leu	Asn	100
ATT	TGT	GAA	GAA	GAC	AGT	TAC	CTG	GCA	GGG	ACG	CTG	GGA	CTC	TCA	GCA	GAT	CAA	ACA	TCT	GGA	AAT	TAC	CTT	AAT	317
Met	Gln	Asp	Ser	Gln	Gly	Val	Leu	Ser	Ser	Phe	Pro	Ala	Pro	Gln	Ala	Val	Gln	Asp	Asn	Pro	Ala	Met	Pro	Thr	125
ATG	CAA	GAC	TCT	CAA	GGA	GTA	CTT	TCT	TCC	TTT	CCA	GCT	CCT	CAG	GCA	GTG	CAG	GAC	AAC	CCA	GCT	ATG	CCC	ACA	392
Ser	Ser	Gly	Ser	Glu	Gly	Asn	Val	Lys	Leu	Cys	Ser	Leu	Glu	Glu	Ala	Gln	Arg	Ile	Trp	Lys	Gln	Lys	Ser	Ala	150
TCC	TCA	GGC	TCA	GAA	GGG	AAT	GTC	AAG	CTT	TGC	TCC	CTA	GAA	GAA	GCT	CAA	AGG	ATA	TGG	AAA	CAA	AAG	TCG	GCA	467
Glu	Ile	Tyr	Pro	Ile	Met	Asp	Lys	Ser	Ser	Arg	Thr	Arg	Leu	Ala	Leu	Ile	Ile	Cys	Asn	Glu	Glu	Phe	Asp	Ser	175
GAG	ATT	TAT	CCA	ATA	ATG	GAC	AAG	TCA	AGC	CGC	ACA	CGT	CTT	GCT	CTC	ATT	ATC	TGC	AAT	GAA	GAA	TTT	GAC	AGT	542
Ile	Pro	Arg	Arg	Thr	Gly	Ala	Glu	Val	Asp	Ile	Thr	Gly	Met	Thr	Met	Leu	Leu	Gln	Asn	Leu	Gly	Tyr	Ser	Val	200
ATT	CCT	AGA	AGA	ACT	GGA	GCT	GAG	GTT	GAC	ATC	ACA	GGC	ATG	ACA	ATG	CTG	CTA	CAA	AAT	CTG	GGG	TAC	AGC	GTA	617
Asp	Val	Lys	Lys	Asn	Leu	Thr	Ala	Ser	Asp	Met	Thr	Thr	Glu	Leu	Glu	Ala	Phe	Ala	His	Arg	Pro	Glu	His	Lys	225
GAT	GTG	AAA	AAA	AAT	CTC	ACT	GCT	TGC	GAC	ATG	ACT	ACA	GAG	CTG	GAG	GCA	TTT	GCA	CAC	CGC	CCA	GAG	CAC	AAG	692
Thr	Ser	Asp	Ser	Thr	Phe	Leu	Val	Phe	Met	Ser	His	Gly	Ile	Arg	Glu	Gly	Ile	Cys	Gly	Lys	Lys	His	Ser	Glu	250
ACC	TCT	GAC	AGC	ACG	TTC	CTG	GTG	TTC	ATG	TCT	CAT	GGT	ATT	CGG	GAA	GGC	ATT	TGT	GGG	AAG	AAA	CAC	TCT	GAG	767
Gln	Val	Pro	Asp	Ile	Leu	Gln	Leu	Asn	Ala	Ile	Phe	Asn	Met	Leu	Asn	Thr	Lys	Asn	Cys	Pro	Ser	Leu	Lys	Asp	275
CAA	GTG	CCA	GAT	ATA	CTA	CAA	CTC	AAT	GCA	ATC	TTT	AAC	ATG	TTG	AAT	ACC	AAG	AAC	TGC	CCA	AGT	TTG	AAG	GAC	842
Lys	Pro	Lys	Val	Ile	Ile	Ile	Gln	Ala	Cys	Arg	Gly	Asp	Ser	Pro	Gly	Val	Val	Trp	Phe	Lys	Asp	Ser	Val	Gly	300
AAA	CCG	AAG	GTG	ATC	ATC	ATC	CAG	GCC	TGC	CGT	GGT	GAC	AGC	CCT	GGT	GTG	GTG	TGG	TTT	AAA	GAT	TCA	GTA	GGA	917
Val	Ser	Gly	Asn	Leu	Ser	Leu	Pro	Thr	Thr	Glu	Glu	Phe	Glu	Asp	Asp	Ala	Ile	Lys	Lys	Ala	His	Ile	Glu	Lys	325
GTT	TCT	GGA	AAC	CTA	TCT	TTA	CCA	ACT	ACA	GAA	GAG	TTT	GAG	GAT	GAT	GCT	ATT	AAG	AAA	GCC	CAC	ATA	GAG	AAG	992
Asp	Phe	Ile	Ala	Phe	Cys	Ser	Ser	Thr	Pro	Asp	Asn	Val	Ser	Trp	Arg	His	Pro	Thr	Met	Gly	Ser	Val	Phe	Ile	350
GAT	TTT	ATC	GCT	TTC	TGC	TCT	TCC	ACA	CCA	GAT	AAT	GTT	TCT	TGG	AGA	CAT	CCC	ACA	ATG	GGC	TCT	GTT	TTT	ATT	1067
Gly	Arg	Leu	Ile	Glu	His	Met	Gln	Glu	Tyr	Ala	Cys	Ser	Cys	Asp	Val	Glu	Glu	Ile	Phe	Arg	Lys	Val	Arg	Phe	375
GGA	AGA	CTC	ATT	GAA	CAT	ATG	CAA	GAA	TAT	GCC	TGT	TCC	TGT	GAT	GTG	GAG	GAA	ATT	TTC	CGC	AAG	GTT	CGA	TTT	1142
Ser	Phe	Glu	Gln	Pro	Asp	Gly	Arg	Ala	Gln	Met	Pro	Thr	Thr	Glu	Arg	Val	Thr	Leu	Thr	Arg	Cys	Phe	Tyr	Leu	400
TCA	TTT	GAG	CAG	CCA	GAT	GGT	AGA	GCG	CAG	ATG	CCC	ACC	ACT	GAA	AGA	GTG	ACT	TTG	ACA	AGA	TGT	TTC	TAC	CTC	1217
Phe	Pro	Gly	His	End																					404
TTC	CCA	GGA	CAT	TAA	AATAAGGAAACTGTATGAATGTCGCGGGCAGGAAGTGAAGAGATCGTTCTGTAAAAGGTTTTTGGAAATTATGTCTGCT																			1311	
GAATATAAATTTTTTGAATATAAATCTGGTAGAAAAATGAAAAAAAAAAAAAAAAAAAA—3'																								1374	

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02339

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12N 9/64, 15/57; C07K 7/00; A61K 37/02 U.S. Cl.: 435/226, 320.1; 530/330; 536/27; 514/17						
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; padding: 5px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">U. S.</td> <td style="padding: 5px;">435/219, 226, 320.1; 530/330, 345, 351; 536/277; 514/17</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U. S.	435/219, 226, 320.1; 530/330, 345, 351; 536/277; 514/17
Classification System	Classification Symbols					
U. S.	435/219, 226, 320.1; 530/330, 345, 351; 536/277; 514/17					
Computer Search - CA and APS						
III. DOCUMENTS CONSIDERED TO BE RELEVANT *						
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³				
Y	US, A, 4,636,492 (Kettner, et. al.), 13 January 1987, see entire patent.	14-22, 28				
Y	US, A, 4,644,055 (Kettner, et. al.), 17 February 1987, see entire patent.	14-22, 28				
Y	J. Biol. Chem., Volume 263, No. 19, issued 05 July 1988, R. A. Black, et. al., "Generation Of Biologically Active Interleukin-1 β By Proteolytic Cleavage Of The Inactive Precursor", see pages 9437-9442, see particularly page 9439, column 2, line 42 - page 9440, column 1, line 1 and page 9441, column 1, line 25-27.	1-22, 28, 30				
Y	Proc. Natl. Acad. Sci., USA, Volume 86, issued July 1989, M. J. Kostura, et. al., "Identification Of A Monocyte Specific Pre-interleukin 1 β Convertase Activity", see pages 5227-5231.	1-22, 28, 30				
X — Y	FEBS Letts., Volume 247, No. 2, issued 24 April 1989, R. A. Black, "Activation Of Interleukin-1 β By A Co-induced Protease", see pages 386-390.	1-4 5-22, 28, 30				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report				
17 July 1991		26 JUL 1991				
International Searching Authority		Signature of Authorized Officer				
TSA/US		 Charles F. Peterson				

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: 1-22, 28, 30. Examiner agrees with applicant that claim 22 should be part of Group III and therefore has been examined.
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without unduly multiplying the additional fee, the International Searching Authority:

Remark on Protest

- ☐ The additional search fees were accompanied by the protest
- ☐ No protest accompanied the payment of additional search fees

Group I, claims 1-4, drawn to a protease molecule.

Group II, claims 5-13, drawn to a DNA sequence, an expression vector containing the DNA, and a method of using the expression vector to make a protease.

Group III, claims 14-22 and 28, drawn to an inhibitor, a pharmaceutical composition comprising the inhibitor and a method of use.

Group VI, claim 23, drawn to a method for improving wound healing.

Group VII, claim 24, drawn to a method for treating arthritis.

Group VIII, claims 25-26, drawn to a method for treating an autoimmune disease.

Group IX, claim 27, drawn to a method for reducing the side effects of radiation.

Group X, claim 29, drawn to a method of treating inflammation.

Group XI, claim 29, drawn to a method of treating an autoimmune disease.

Group XII, claim 30, drawn to an antisense oligonucleotide.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	J. Biol. Chem., Volume 264, No. 10, issued 05 April 1989, R. A. Black, et. al., "A Pre-aspartate-specific Protease From Human Leukocytes That Cleaves Pro-interleukin-1 β ", see pages 5323-5326, see particularly page 5324, last full sentence and page 5326, line 8-13.	1-22,28,30
Y	Nature, Volume 315, issued 20 June 1985, C. J. March, et. al., "Cloning, Sequence And Expression Of Two Distinct Human Interleukin-1 Complementary DNA's", see pages 641-647, see particularly sentence spanning pages 644-645 and first full sentence on page 646.	1-22,28,30
Y	Proc. Natl. Acad. Sci., USA, Volume 78, No. 11, issued November 1981, S. V. Suggs, et. al., "Use Of Synthetic Oligonucleotides As Hybridization Probes: Isolation Of Cloned cDNA Sequences For Human β_2 -microglobulin", see pages 6613-6617.	1-22,28,30

